SUPPLEMENTAL FIGURE LEGENDS

Figure S1. RADX prevents MUS81-dependent DSBs (related to Figure 2). (A) Immunoblot to validate RADX siRNAs. (B) Representative images of U2OS cells transfected with non targeting siRNA (siNT) or siRNA targeting the RADX gene (siRADX) treated with 3mM HU for 4 hours and stained for BrdU and γH2AX. (C) Representative images of the neutral comet assay performed in the absence of exogenous damage. (D) Cell cycle profiles of U2OS cells transfected with the indicated siRNAs were analyzed by flow cytometry using BrdU and propidium iodide staining. (E) Immunoblot to validate the RADX null cells obtained by CRISPR-CAS editing of the gene in U2OS and 293T cells (arrow points to RADX, *non-specific band). The non specific band is not observed in other blots because of better separation on lower percentage gels. (F) Immunoblot of RADX expression levels in U2OS and RADXΔ cells before and after complementation. (G) Immunoblot depicting knockdown of MUS81 and RADX. (H) DSBs in RADX wild-type and RADXΔ cells were measured by neutral comet assay 72 hours after transfection with the indicated siRNAs. P-values were calculated with a Kruskal-Wallis ANOVA and Dunn's multiple comparison post-test. The number of comet tails analyzed in each sample is indicated in parentheses. Experiment is representative of two replicates. Immunoblot to confirm knockdown of MUS81 is shown.

Figure S2. Multiple sequence alignment of RADX and RPA70 (related to Figure 3)**.** Multiple sequence alignment of RPA70 and RADX from the indicated species was completed with CLUSTALW2.

Figure S3. RADX binds DNA through its RPA70-like OB-2 fold domain (related to Figure 3)**.** (A) Coomassie stained SDS-PAGE gels of purified RADX from insect cells. Mass spectrometry indicated that the smaller molecular weight band is a RADX degradation product. (B)

Immunoblot depicting the complementation of RADX-proficient (WT) U2OS and RADXΔ cells with WT or OB-mutant RADX. Note that lanes 1,2,4, and 5 are also presented in Figure S1D. (C) Sequence alignment of RPA70A and OB-2 of RADX. The cyan, blue and green dots indicate residues involved in hydrogen bonds, salt bridges and pi stacking interactions with DNA as observed in the crystal structure of RPA70AB in complex with DNA. (D) Ribbon representations of the RPA70-ssDNA complex (left, blue/cyan) extracted from the crystal structure of RPA70AB-dC8 (PDB code 1JMC) and a RADX OB-2 homology model (right, salmon). Residues selected for inclusion in the OBm mutant based on the model are highlighted.

Figure S4. RADX overexpression suppresses RAD51 foci formation and causes DNA damage (related to Figures 4 and 5)**.** (A and B) Immunoblots after transfection with the indicated siRNAs in wild-type or RADXΔ U2OS cells**.** (C) U2OS cells transfected with non-targeting (NT) or RADX siRNA were labeled with 10 µM BrdU for 22 hours prior to treatment with 3 mM HU for the indicated times. The cells were fixed and stained for BrdU using non-denaturing conditions to measure the amount of ssDNA. The mean+/-SEM from a representative experiment are depicted (n=107 per sample). (D) U2OS cells transfected with non-targeting (NT) or RADX siRNA were left untreated or treated with 3mM HU for 24 hours. The soluble and chromatin fractions were immunoblotted for the indicated proteins. (E) Mock or Flag-RADX overexpressing U2OS cells were labeled with 10µM EdU for thirty minutes prior to staining for EdU and RADX. The percentage of EdU positive cells is presented (mean+/-SEM, n=3). P-value derived from a Mann Whitney test. (F) Cell cycle profiles of mock and GFP-RADX overexpressing U2OS cells were analyzed by propidium iodide staining and flow cytometry. (G) Mock and GFP-RADX overexpressing U2OS cells were stained for γH2AX. The mean+/-SEM from a representative experiment is presented (n=144). P-value was derived from a Mann Whitney test. All representative experiments were repeated at least twice.

Figure S5. RADX deletion causes excessive RAD51 activity at forks and restores fork protection without restoration of HR in BRCA2/RAD51-compromised cells (related to Figures. 5, 6 and 7)**.** (A-D) Immunoblots depicting RADX, BRCA2, SMARCAL1 or ZRANB3 knockdown as indicated. (E) Summary of the median CldU/IdU ratio and number of fibers analyzed for the single-molecule nascent strand degradation assay depicted in Figure 6. (F) The percentage of GFP-positive DR-GFP-U2OS cells after transfection with the indicated siRNAs and I-SceI expression vector was measured by flow cytometry. The concentration indicates the amount of RAD51 siRNA used in each sample. The mean and SD from three experiments in which 25,000 cells were scored is depicted. (G) Mock and GFP-RADX overexpressing U2OS cells were irradiated (3 Gy) and then allowed to recover for the indicated times before staining for RAD51. The number of cells with >10 RAD51 foci after detergent extraction is presented (Mean+/-SEM from n=3). P value derived from a two-way ANOVA with Sidak's post test for multiple comparisons. (H) The percentage of GFP-positive DR-GFP-U2OS cells after transfection with the indicated vectors was measured by flow cytometry. The mean and SEM from three experiments in which 25,000 cells were scored is depicted. (I) Parental or RADXΔ U2OS cells transfected with the BRCA2 siRNAs were either untreated or treated with 2Gy**.** Surviving cell colonies were stained and scored. (mean+/-SD, n=3). The experiment was completed in triplicate.

Figure S6. RADX and RAD51 expression correlations with patient outcome (related to Figure 6)**.** (A-D) Kaplan Meier plots of RADX and RAD51 mRNA expression and correlation with patient outcome in breast and lung cancers were derived from KM plotter (Szász et al. 2016). Settings for all graphs were as follows: auto select best cutoff, only JetSet best probe, remove redundant samples, and exclude biased arrays.

Figure S7. Model for RADX function (related to Figures 1-7)**.** (A) In unstressed cells, RADX prevents inappropriate RAD51-dependent fork reversal. In RADXΔ cells, RAD51 accumulates at forks causing fork cleavage by the MUS81 endonuclease. (B) A balance of RAD51 activity is maintained at stalled replication forks by RADX and BRCA2. In BRCA2-mutant cells, there is insufficient RAD51 to prevent MRE11-dependent fork degradation. However, deletion of RADX restores fork protection and also yields decreased sensitivity to PARP inhibitors. See text for more details.

Figure S1 (related to Figure 2)

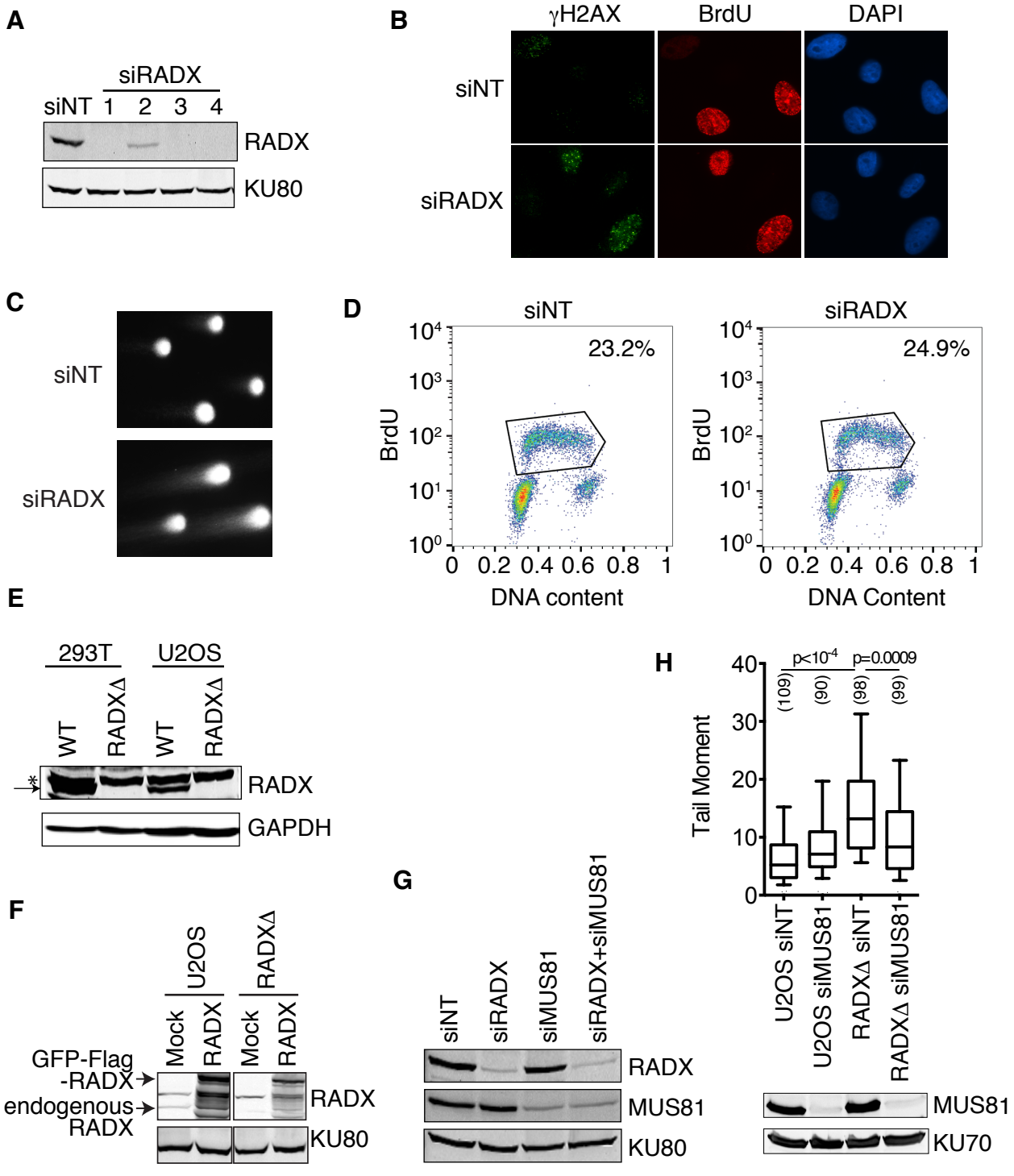


Figure S2 (related to Figure 3)

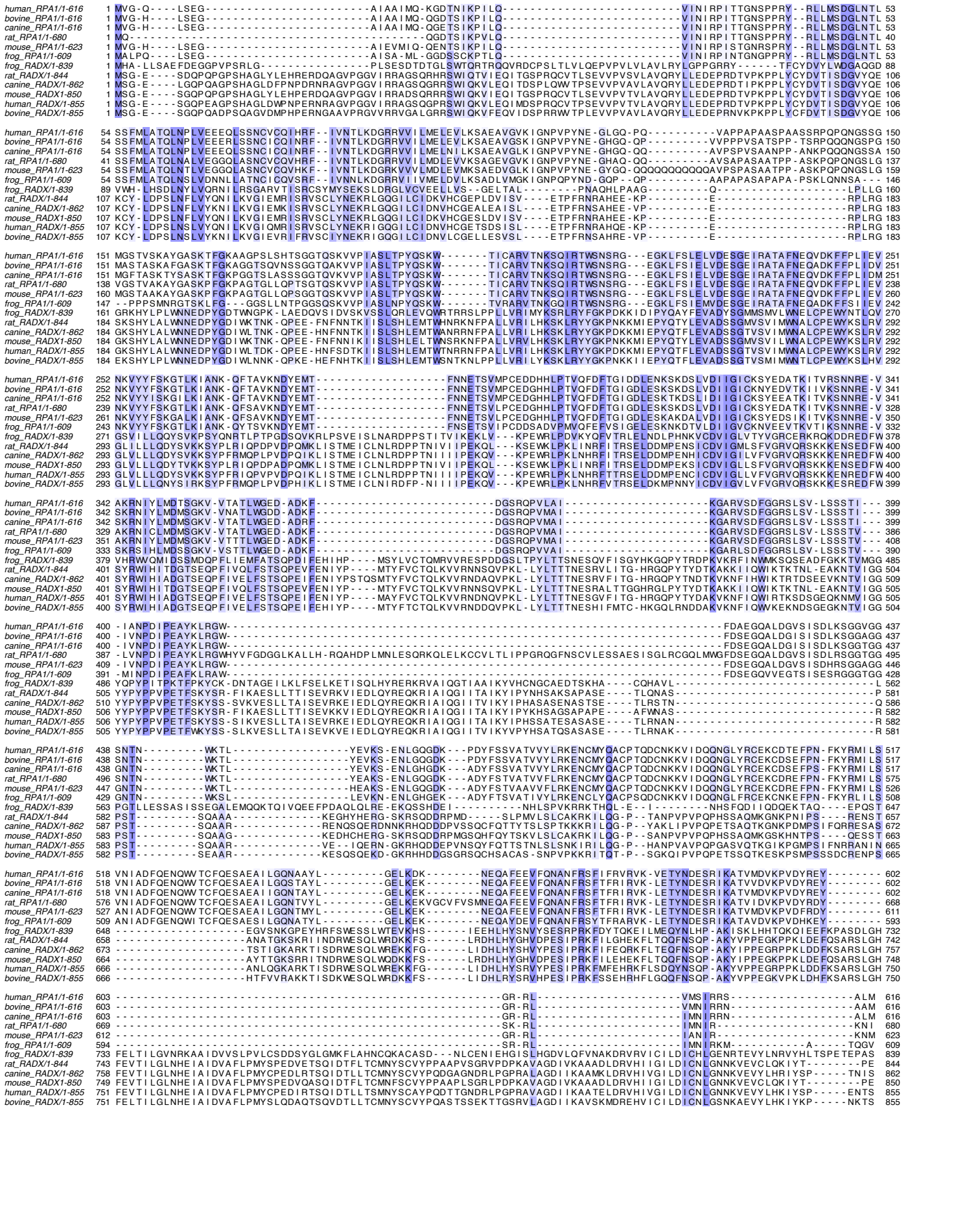


Figure S3 (related to Figure 3)

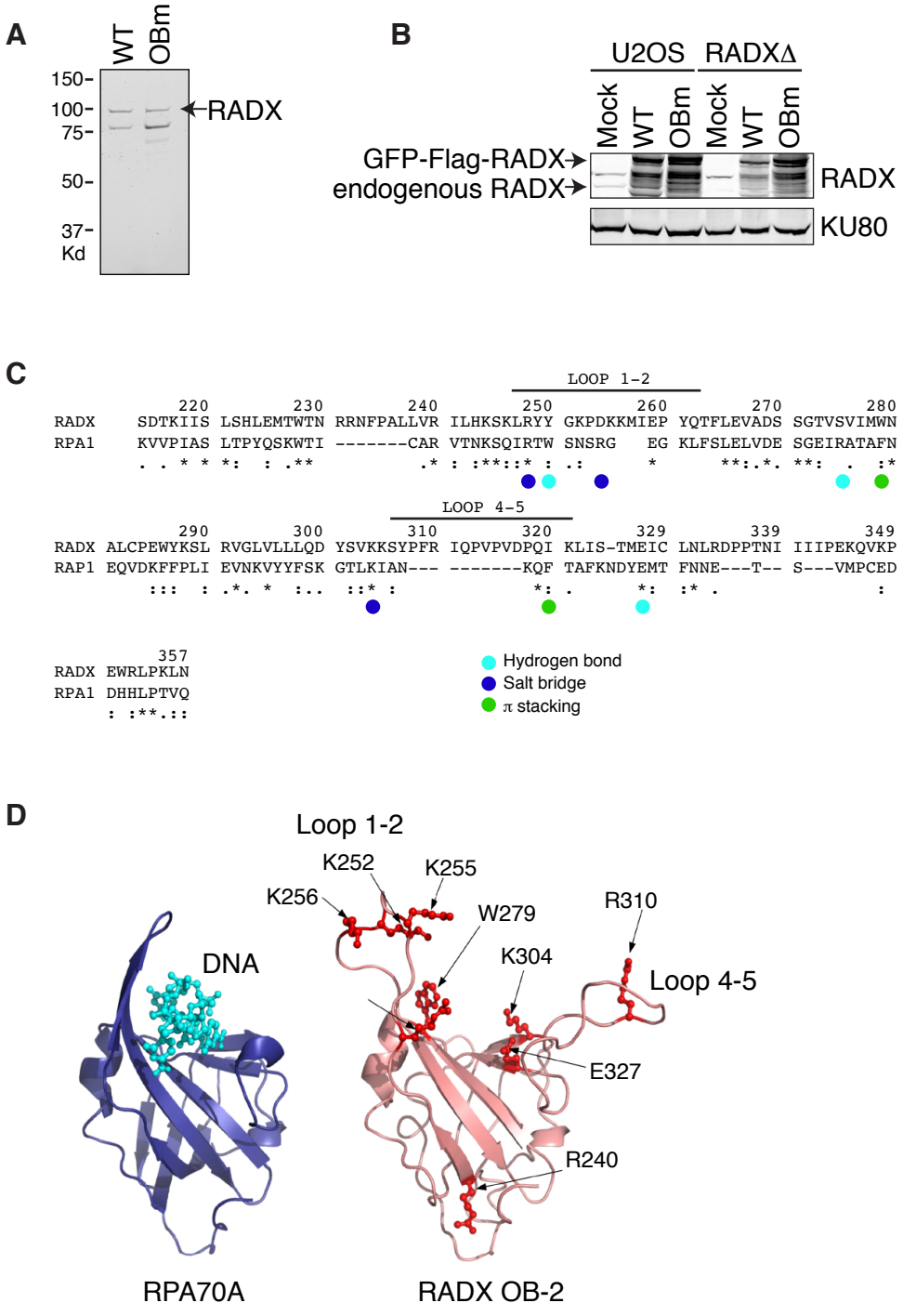


Figure S4 (related to Figures 4 and 5)

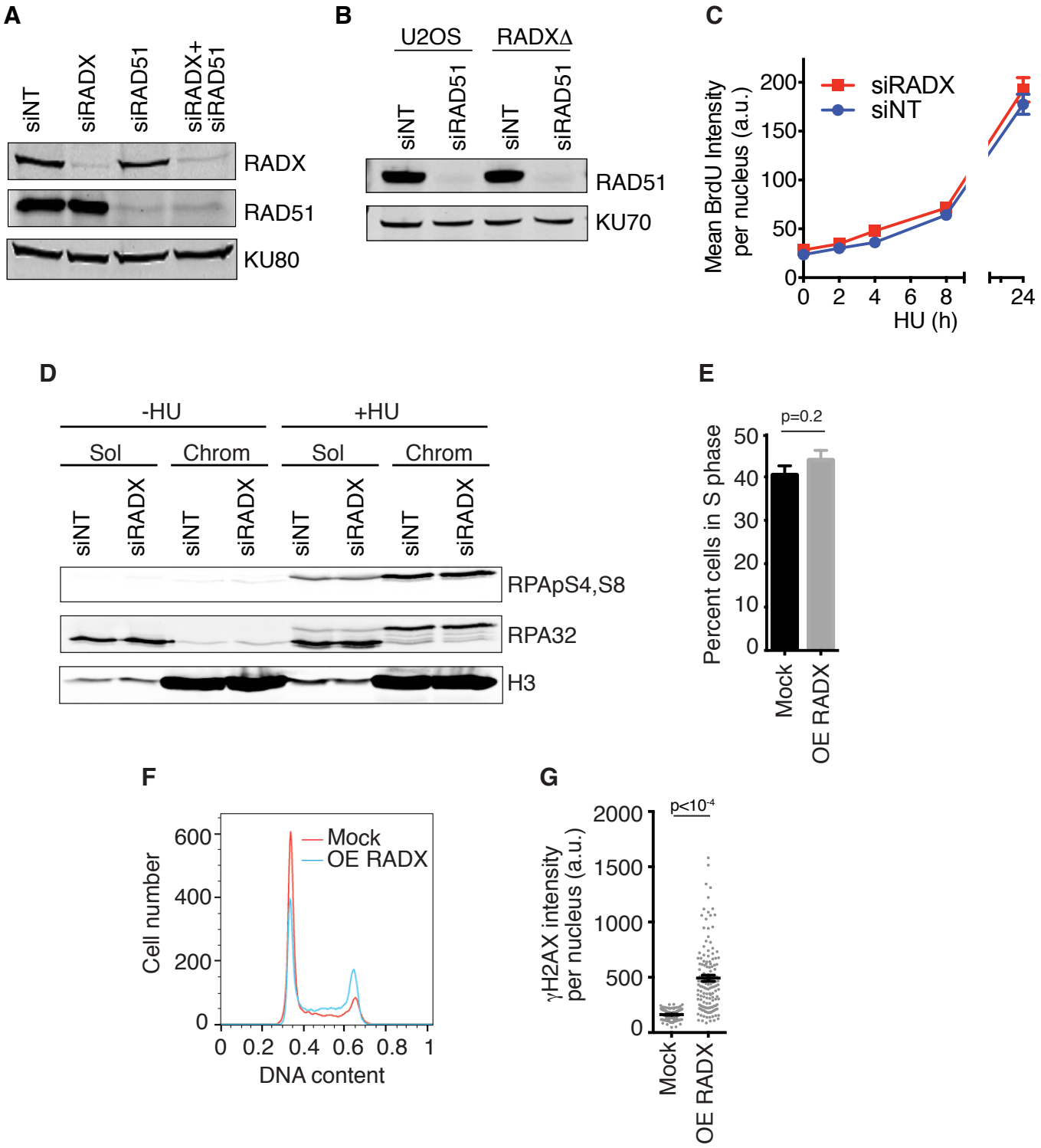
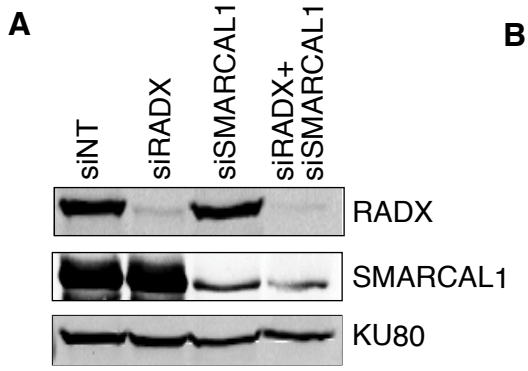
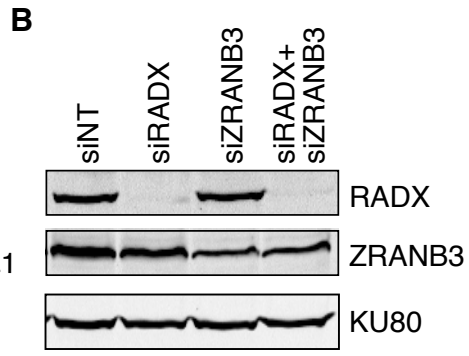
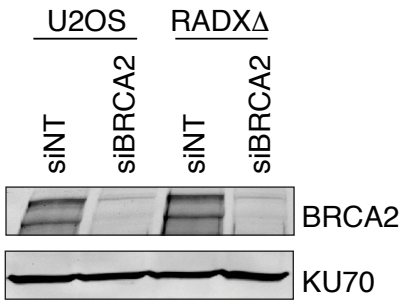
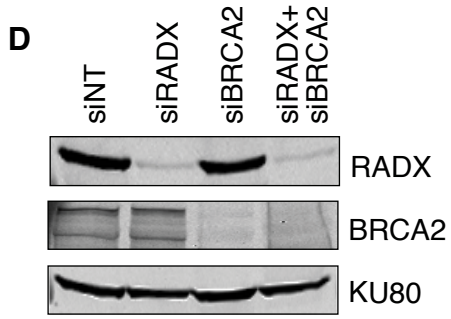


Figure S5 (related to Figures 5-7)



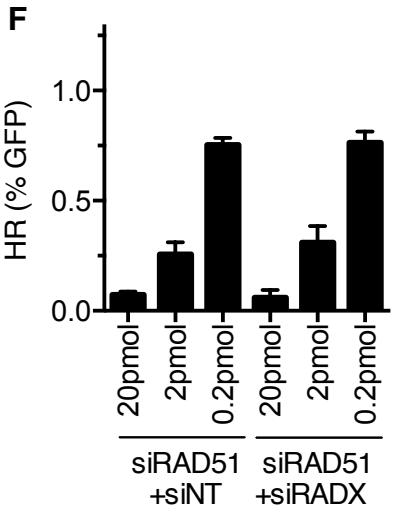


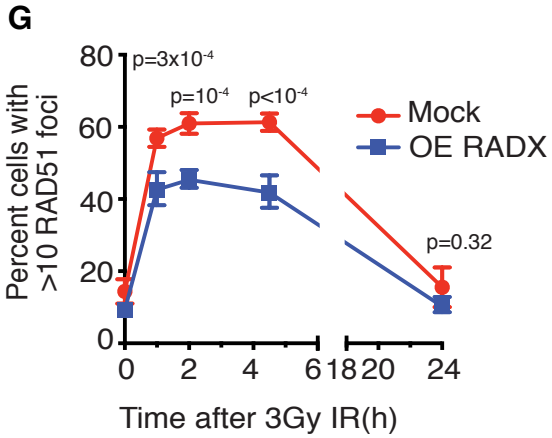


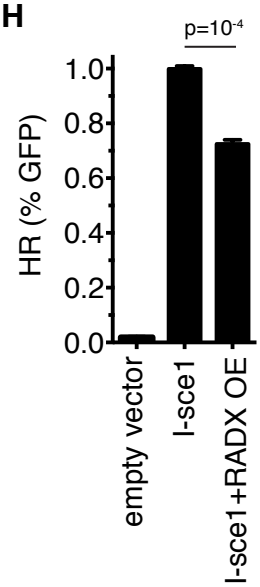


E

Figure	Sample	Median CldU/IdU ratio	Number of fibers analyzed
Fig. 6A	U2OS siNT	0.93	101
	U2OS siRADX	0.91	111
	U2OS siBRCA2	0.67	112
	U2OS siRADX+siBRCA2	0.86	105
Fig. 6B	CAPAN-1 siNT	0.78	203
	CAPAN-1 siRADX	0.99	204
Fig. 6C	U2OS Mock	1.176	232
	U2OS RADX OE	0.87	243







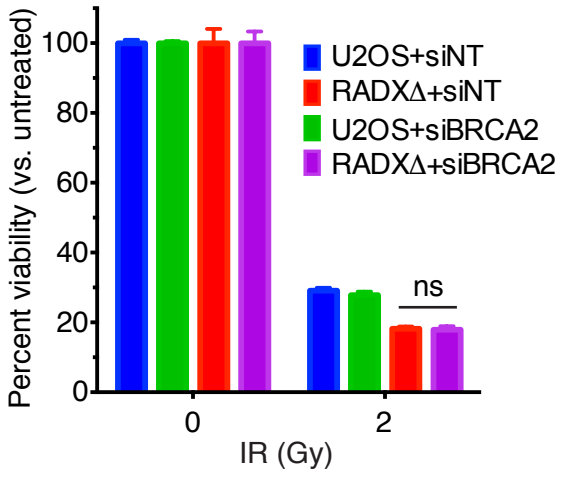


Figure S6 (related to Figure 7)

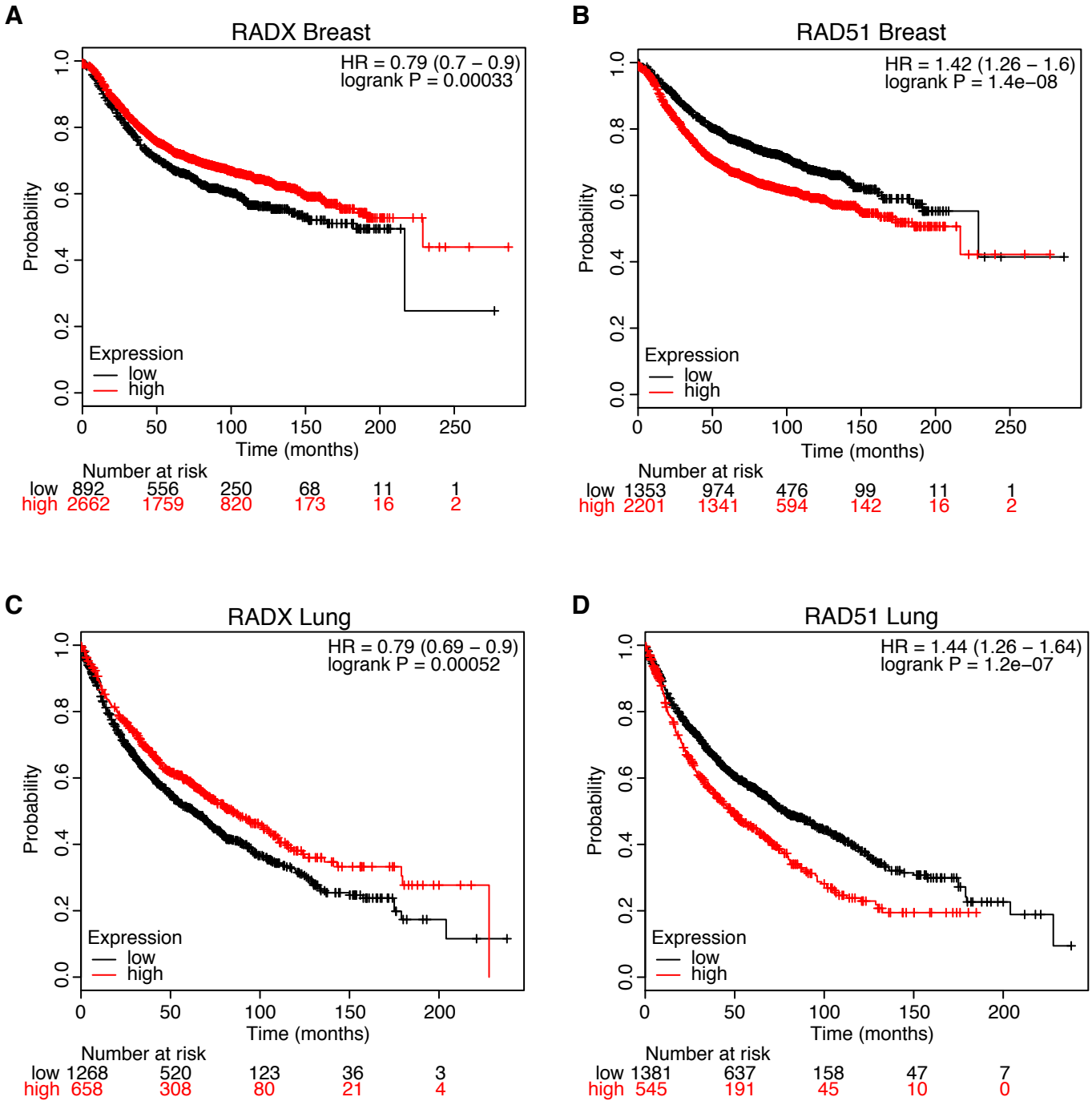
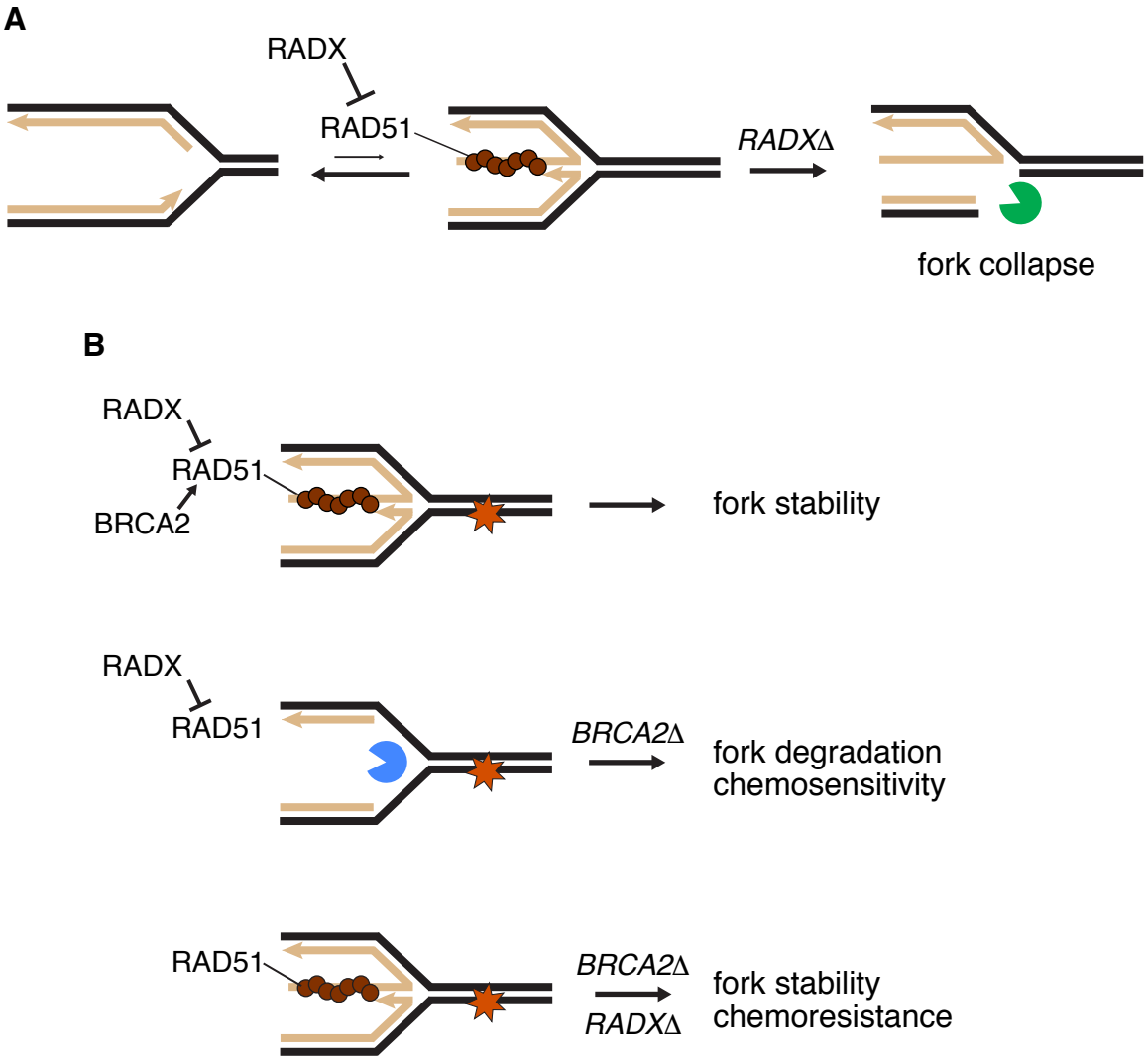


Figure S7 (related to Figures 1-7)



SUPPLEMENTAL ITEM

Table S2. List of oligonucleotides used in this study (Related to STAR METHODS: Key Resources Table)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
All stars negative control siRNA (siNT)	Qiagen	Cat#SI03650318
siRADX#1: GAACACAACUUUAGCGAUA	ON-TARGETplus Dharmacon	Cat#J-014634-17
siRADX#2: CUUCAGAAAUAGAGCGCAC	ON-TARGETplus Dharmacon	Cat#J-014634-19
siRADX#3: GCUUGAACUCUCUCGUAUA	ON-TARGETplus Dharmacon	Cat#J-014634-20
siRADX#4: CAUAGAGGCCAGCCGUAUA	ON-TARGETplus Dharmacon	Cat#J-014634-21
siMUS81: GGGUAUACCUGGUGGAAGA	siGENOME Dharmacon	Cat#D-016143-04
siRAD51#1: AAGGGAATTAGTGAAGCCAAA	Qiagen Flexitube	Cat#SI02663682
siRAD51#2: CAGGTGGTAGCTCAAGTGGAT	Qiagen Flexitube	Cat#SI03072272
siBRCA2: CAGGACACAATTACAACTAAA	Qiagen Flexitube	Cat#SI02653434
siSMARCAL1: GCUUUGACCUUCUUAGCAA	ON-TARGETplus Dharmacon	Cat#J-013058-06
siZRANB3: CAAGAGAUAUCAUCGAUUAtt	Ambion Silencer Select	Cat#4392420
siBLM#1: GAGCACAUCUGUAAAUUAA	siGENOME Dharmacon	Cat#D-007287-01
siBLM#2: GAGAAACUCACUUCAAUAA	siGENOME Dharmacon	Cat#D-007287-03
siBLM#3: CAGGAUGGCUGUCAGGUUA	siGENOME Dharmacon	Cat#D-007287-04
siBLM#4: CUAAAUCUGUGGAGGGUUA	siGENOME Dharmacon	Cat#D-007287-05
Guide RNA RADX#1: 3'-CACCGAATCAAAACTGCGATACTA-5'	This paper	NA
Guide RNA RADX#2: 3'-CACCGTTACCATTACATGTTAAAC-5'	This paper	NA
RADXmRNA(Mouse) Forward primer 5'-TTGGAGCACCCCGAAAGGGATCAGG	This paper	NA
RADXmRNA(Mouse) Reverse primer: 5' ATCTAGGGACTCCCCACAGTGGACC	This paper	NA